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PLoS one

Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules

Xiaoyu Wen[1☯], Takayuki Abe[1☯], Hiroshi Kukihara[1], Shuhei Taguwa[1], Yoshio Mori[1], Hideki Tani[1], Nobuyuki Kato[2], Tetsuro Suzuki[3], Masashi Tatsumi[4], Kohji Moriishi[1], Yoshiharu Matsuura[1]*

1 Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, **2** Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, **3** Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan, **4** AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Abstract

To eliminate hepatitis C virus (HCV) from infected hepatocytes, we generated two therapeutic molecules specifically activated in cells infected with HCV. A dominant active mutant of interferon (IFN) regulatory factor 7 (IRF7) and a negative regulator of HCV replication, VAP-C (Vesicle-associated membrane protein-associated protein subtype C), were fused with the C-terminal region of IPS-1 (IFNβ promoter stimulator-1), which includes an HCV protease cleavage site that was modified to be localized on the ER membrane, and designated cIRF7 and cVAP-C, respectively. In cells expressing the HCV protease, cIRF7 was cleaved and the processed fragment was migrated into the nucleus, where it activated various IFN promoters, including promoters of IFNα6, IFNβ, and IFN stimulated response element. Activation of the IFN promoters and suppression of viral RNA replication were observed in the HCV replicon cells and in cells infected with the JFH1 strain of HCV (HCVcc) by expression of cIRF7. Suppression of viral RNA replication was observed even in the IFN-resistant replicon cells by the expression of cIRF7. Expression of the cVAP-C also resulted in suppression of HCV replication in both the replicon and HCVcc infected cells. These results suggest that delivery of the therapeutic molecules into the liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

Citation: Wen X, Abe T, Kukihara H, Taguwa S, Mori Y, et al. (2011) Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules. PLoS ONE 6(1): e15967. doi:10.1371/journal.pone.0015967

Editor: Paul Digard, University of Cambridge, United Kingdom

Received September 29, 2010; **Accepted** December 7, 2010; **Published** January 6, 2011

Funding: This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Osaka University Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: matsuura@biken.osaka-u.ac.jp

☯ These authors contributed equally to this work.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma [1,2]. Although the proportion of patients achieving a sustained virological response (SVR) has been increased by the recent used of combination therapy with pegylated-interferon-α (PEG-IFNα) and ribavirin (RBV), half of patients still exhibit no response to this therapy, suggesting that the IFN signaling pathway is modulated by HCV infection. In addition, various side effects have been reported in more than 20% of patients treated with this combination therapy [3].

HCV belongs to the family *Flaviviridae* and possesses a single positive-stranded RNA genome that encodes a single polyprotein composed of about 3,000 amino acids. The HCV polyprotein is processed into 10 viral proteins by host and viral proteases. Viral structural proteins, including the capsid protein and two envelope proteins, are located in the N-terminal one third of the polyprotein, followed by nonstructural proteins. The NS2 protease cleaves its own carboxyl terminus and NS3 cleaves the downstream positions to produce NS4A, NS4B, NS5A and NS5B. Although laboratory strains of HCV propagating in cell culture (HCVcc) have been established based on the full-length genome of the genotype 2a JFH1 strain [4], establishment of a robust cell culture system capable of propagating serum-derived HCV from hepatitis C patients has not yet been achieved.

Type I IFN exhibits potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs) which encode proteins involved in the establishment of antiviral state in cells [5]. IFNs induce transcription of ISGs through activation of the Jak-STAT pathway [6]. Binding of type I IFN to the IFN receptor induces phosphorylation of the receptor-associated tyrosine kinases, Jak1 and Tyk2, and then these kinases activate STAT1 and STAT2. The phosphorylated STATs migrate into the nucleus and activate ISG promoters through binding to the specific responsible elements. HCV infection has been suggested to impair the IFN production through multiple pathways. The IFN-induced Jak-STAT signaling is inhibited in cells and transgenic mice expressing HCV proteins and in the liver biopsy samples of chronic hepatitis C patients [7–9].

Induction of type I IFN upon infection with pathogens is crucial for innate immunity, and it is mediated by the activation of pattern-recognition receptors, including Toll-like receptors (TLRs) and cytosolic receptors, such as RIG-I and MDA5 [10–12]. The induction of type I IFN is primarily controlled at the gene transcriptional level, wherein a family of transcription factors known as IFN regulatory factors (IRFs) play a pivotal role. IRF3

Promising Targets for Anti-Hepatitis C Virus Agents

T. Yosida, M. Kondoh* and K. Yagi*

Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

Abstract: Hepatitis C virus (HCV) infection is a serious global health problem, with 3-4 million new cases reported each year. Chronic HCV infection places 170 million people at risk of developing liver cirrhosis and hepatocellular carcinoma. However, difficulties in preparing HCV particles *in vitro* have delayed development of effective anti-HCV therapies. In 2005, Wakita *et al.* developed an *in vitro* method to prepare HCV particles, thereby enabling researchers to better understand the mechanism of HCV infection. Other recent advances include development of a virus-free system for evaluating HCV replication and the identification of HCV receptors, such as claudin-1 and occludin, that may serve as targets for anti-HCV drugs. In this review, we discuss recent findings in HCV infection research, including discovery of new potential targets for anti-HCV therapy.

Keywords: Hepatitis c virus, CD81, claudin-1, NS3 helicase, cyclophilin, miRNA122.

INTRODUCTION

It is estimated that approximately 170 million people worldwide are infected with hepatitis C virus (HCV). Chronic HCV infection induces cirrhosis of the liver or hepatocellular carcinoma. Currently, no vaccines or inhibitors that block HCV entry into cells are approved for clinical use. Standard therapy for chronic HCV infection is the combination of pegylated interferon (IFN) and ribavirin (RBV); however, only 50% to 60% of infected patients get a sustained anti-viral response by this therapy. In addition, the severe side effects typical of IFN and RBV treatment often lead patients to stop treatment, and development of novel treatments with fewer serious side effects are therefore necessary.

Hepatitis C virus is a single-stranded RNA virus belonging in the family *Flaviviridae*. The viral genome is approximately 9,600 nucleotides, containing a 5'untranslated region (5' UTR), a region encoding a polyprotein of about 3,000 amino acids, and a 3' UTR. An internal ribosome-entry site (IRES) in the 5' UTR induces cap-independent translation. Once translated, the viral polyprotein is proteolytically processed by cellular signal peptidases and viral proteases into at least 10 mature viral proteins. Three of these proteins (Core, E1, and E2) are the structural proteins included in virions. It is unclear whether protein p7 is included in virions. Non-structural proteins (NS) include NS2, NS3, NS4A, NS4B, NS5A, and NS5B, and all except NS2 are necessary for formation of the complex associated with viral replication. In this review, we summarize recent developments in anti-HCV agents and discuss potent targets for anti-HCV agents.

INHIBITORS OF HCV ENTRY INTO CELLS

HCV Receptors

Hepatitis C virus contains two glycosylated envelope proteins, E1 and E2. While the role of E1 in infection is poorly understood, E2 is known to play a critical role through binding to the cell surface receptor and facilitating virus entry. Several receptors and co-receptors are involved in HCV infection, including CD81, scavenger receptor class B type I (SR-BI), low-density lipoprotein receptor (LDLR), claudin-1, and occludin [1-5]. Although it has been demonstrated that both CD81 and SR-BI directly bind to E2, there is no evidence that claudin-1 and occludin bind the HCV envelope, suggesting that claudin-1 and occludin may interact with other co-receptors to induce HCV entry.

Development of inhibitors that block envelope protein E2 from interacting with cellular receptors is an important area of anti-HCV research. One such class of inhibitors, (ssDNA) aptamers that recognize the HCV E2 protein, was isolated using a living cell surface technique (Systematic Evolution of Ligands by Expotential Enrichment). The ssDNA aptamer ZE2 binds to E2 with high affinity and inhibits its interaction with CD81, and was shown to block HCV infection *in vitro* [6].

Other inhibitors of HCV infection include proteins that bind to or modulate the activity of CD81 and prevent its interaction with CD81 with E2. Salicylate derivatives identified through virtual screening inhibit HCV infection by binding to the open conformation of the large extracellular loop (LEL) of CD81 and preventing its binding to E2. Benzyl salicylate inhibits the interaction of CD81-LEL with E2 by 25% at 50 μM [7]. Another modulator of CD81 activity is PSCK9, a regulator protein of membrane-bound receptors such as LDLR, ApoER2, and very low-density lipoprotein receptor. A recent study showed that PSCK9 deregulates the cell surface localization of CD81. Soluble PCSK9 inhibits HCV infection *in vitro* in a dose-dependent manner [8].

Claudin-1 has been identified as a co-receptor involved in HCV entry into cells, and its interaction with CD81 may help facilitate the early and late stages of HCV entry [4]. Claudin-1 is estimated to be a co-receptor that interacts with CD81. Recently, a claudin-1 antibody was developed that helped elucidate the role of claudin-1 in HCV infection. Anti-claudin-1 inhibited HCV infection at the same stage of HCV entry at which an anti-CD81 antibody did [9]. Since there is no evidence that claudin-1 binds directly to any HCV envelope proteins, it is believed that claudin-1 interacts with CD81 to form a complex that enables HCV cell entry, and may thus serve as a target for development of new HCV entry inhibitors.

Several HCV entry inhibitors that target neither the HCV envelope proteins nor cellular receptors have also been developed. One such inhibitor is C5A, an amphipathic α-helical peptide derived from the membrane anchor domain of HCV NS5A. C5A prevents initiation and spread of HCV infection by destabilizing virions, and has been shown to destroy the integrity of other viral particles, including other *Flaviviridae* (West Nile virus and dengue virus), some paramyxoviruses, and human immunodeficiency virus [10, 11]. C5A might recognize lipid composition of virus membranes, leading to the antiviral activity of C5A to the other viruses [11].

Arbidol is a broad-spectrum antiviral agent that inhibits virus-induced membrane fusion [12-14]. Arbidol is an effective inhibitor of both hepatitis B and C, as well as a wide range of other viruses, including influenza A and B, parainfluenza virus 3, respiratory syncytial virus, and rhinovirus 14. Other agents that block viral entry into host cells include Peptide 75, a peptide derived from the HCV E2 protein transmembrane domain [15], and the Lamiridosins, compounds extracted from *Lamium album* [16]. While the mechanisms through which these agents act to inhibit viral infectivity are poorly understood, continued research may lead to development of additional novel series of inhibitors.

*Address correspondence to these authors at the Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan; Tel: +81-6-6879-8196; Fax: +81-6-6879-8199; E-mail: masuo@phs.osaka-u.ac.jp

Tel/Fax: +81-6-6879-8195; E-mail: yagi@phs.osaka-u.ac.jp

0929-8673/11 $58.00+.00

Nucleic Acids Research Advance Access published February 8, 2011

Nucleic Acids Research, 2011, 1–8
doi:10.1093/nar/gkr047

Adenovirus vector-mediated assay system for hepatitis C virus replication

Takeshi Yoshida[1], Masuo Kondoh[1,*], Manabu Ojima[1], Hiroyuki Mizuguchi[2], Yoshiaki Yamagishi[1], Naoya Sakamoto[3] and Kiyohito Yagi[1,*]

[1]Laboratory of Bio-Functional Molecular Chemistry, [2]Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka and [3]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

Received April 9, 2010; Revised January 17, 2011; Accepted January 18, 2011

ABSTRACT

The efficient delivery of the hepatitis C virus (HCV) RNA subgenomic replicon into cells is useful for basic and pharmaceutical studies. The adenovirus (Ad) vector is a convenient and efficient tool for the transduction of foreign genes into cells *in vitro* and *in vivo*. However, an Ad vector expressing the HCV replicon has never been developed. In the present study, we developed Ad vector containing an RNA polymerase (pol) I-dependent expression cassette and a tetracycline-controllable RNA pol I-dependent expression system. We prepared a hybrid promoter from the tetracycline-responsive element and the RNA pol I promoter. Ad vector particles coding the hybrid promoter-driven HCV replicon could be amplified, and interferon, an inhibitor of HCV replication, reduced HCV replication in cells transduced with the Ad vector coding HCV replicon. This is the first report of the development of an Ad vector-mediated HCV replicon system.

INTRODUCTION

Hepatitis C virus (HCV) is a member of *Flaviviridae* that contains a 9.6-kb positive-sense RNA genome. A total of 170-million people worldwide are infected with HCV, leading to chronic hepatic inflammation, hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (1). Chronic infection with HCV is a major cause of hepatocellular carcinoma (1). Interferon (IFN) therapy is the gold standard method for HCV patients, but it is effective in only 50% of patients and its use has been limited because of severe side effects (2–4). Additional pharmaceutical therapies are needed to overcome HCV. However, the tropism of HCV is limited to chimpanzees and humans, and the mechanism of HCV infection and replication is not fully understood. The HCV genome encodes a polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (5). An HCV subgenome replicon (called HCV replicon in the present study) consisting of a reporter gene and HCV NS genes has allowed various studies of HCV replication and the development of anti-HCV agents (6–8). The delivery of the HCV genome or HCV replicon is a powerful tool for basic and pharmaceutical research, and the transduction of *in vitro* translated HCV RNA genome is often performed by electroporation. However, a convenient and efficient method to transfer the 9.6-kb HCV RNA genome or the 8–9-kb HCV replicon has never been fully developed.

Transcribed RNAs are classified into rRNAs, mRNAs and short RNAs (tRNAs) in mammalian cells. RNA polymerases differ among the transcribed RNA species: RNA polymerase (pol) I for rRNAs, RNA pol II for mRNA and RNA pol III for short RNAs. RNA pol I transcribes RNA without a 5′-cap structure or a 3′-poly-A tail, and a plasmid vector encoding RNA pol I promoter and terminator has been applied to the development of RNA virus-expression system. For instance, influenza viruses, arenavirus and uukuniemi viruses are generated using RNA pol I-driven expression plasmid vectors coding each segment of negative-sense RNA (9–12). Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to a variety of cell types and tissues *in vitro* and *in vivo* in basic research and clinical therapy. Ad vector can be easily prepared, grown to a high titer, and used to efficiently transfer genes into dividing and non-dividing cells. Furthermore, several types of Ad vectors have been developed to expand their tropism and to increase the size of encoded genes (13,14). Ad vector encoding RNA pol I-driven expression of influenza virus

*To whom correspondence should be addressed. Tel: +81 6 6879 8196; Fax: +81 6 6879 8199; Email: masuo@phs.osaka-u.ac.jp
Correspondence may also be addressed to Kiyohito Yagi. Tel/Fax: +81 6 6879 8195; Email: yagi@phs.osaka-u.ac.jp

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Biochemical and Biophysical Research Communications 416 (2011) 119–124

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection

Takeshi Yoshida [a,1], Kazuo Takayama [b,1], Masuo Kondoh [a,*], Fuminori Sakurai [b], Hideki Tani [c], Naoya Sakamoto [d], Yoshiharu Matsuura [c], Hiroyuki Mizuguchi [b,*], Kiyohito Yagi [a]

[a] Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[b] Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[c] Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
[d] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

ARTICLE INFO

Article history:
Received 31 October 2011
Available online 10 November 2011

Keywords:
Hepatitis C virus
Induced pluripotent stem cell
Hepatocyte
Infection
Replication
Experimental model

ABSTRACT

Host tropism of hepatitis C virus (HCV) is limited to human and chimpanzee. HCV infection has never been fully understood because there are few conventional models for HCV infection. Human induced pluripotent stem cell-derived hepatocyte-like (iPS-Hep) cells have been expected to use for drug discovery to predict therapeutic activities and side effects of compounds during the drug discovery process. However, the suitability of iPS-Hep cells as an experimental model for HCV research is not known. Here, we investigated the entry and genomic replication of HCV in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively. We showed that iPS-Hep cells, but not iPS cells, were susceptible to infection with HCVpv. The iPS-Hep cells expressed HCV receptors, including CD81, scavenger receptor class B type I (SR-BI), claudin-1, and occludin; in contrast, the iPS cells showed no expression of SR-BI or claudin-1. HCV RNA genome replication occurred in the iPS-Hep cells. Anti-CD81 antibody, an inhibitor of HCV entry, and interferon, an inhibitor of HCV genomic replication, dose-dependently attenuated HCVpv entry and HCV subgenomic replication in iPS-Hep cells, respectively. These findings suggest that iPS-Hep cells are an appropriate model for HCV infection.

1. Introduction

Hepatitis C virus (HCV), a hepatotropic member of the *Flaviviridae* family, is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. Approximately 130–200 million people are estimated to be infected with HCV worldwide. Each year, 3–4 million people are newly infected with HCV [1]. Thus, overcoming HCV is a critical issue for the World Health Organization.

HCV contains a positive strand ~9.6 kb RNA encoding a single polyprotein (~3000 aa), which is cleaved by host and viral proteases to form structural proteins (core, E1, E2, and p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1]. These virus proteins might be potent targets for anti-HCV drugs. However, combination therapy with interferon and ribavirin, which often causes severe side-effects leading to treatment termination, has been the only therapeutic choice [2]. Very recently, new direct antiviral agents have been approved or are under clinical trials; these agents include NS3 protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors [2–4]. However, the emergence of drug resistance is a serious problem associated with the use of direct antiviral agents [5].

Host targets are alternative targets for the development of anti-HCV drugs. A liver-specific microRNA (miRNA), miR-122, facilitates the replication of the HCV RNA genome in cultured liver cells [6]. Administration of a chemically modified oligonucleotide complementary to miR-122 results in long-lasting suppression of HCV with no appearance of resistant HCV in chimpanzees [7]. Epidermal

Abbreviations: HCV, hepatitis C virus; iPS-Hep cells, human induced pluripotent stem cells-derived hepatocyte-like cells; HCVpv, HCV pseudotype virus; SR-BI, scavenger receptor class B type I; miRNA, microRNA; EGF-R, epidermal growth factor receptor; EphA2, ephrin factor A2; iPS cells, human induced pluripotent stem cells; FCS, fetal calf serum; Ad, adenovirus; HNF-4α, hepatocyte nuclear factor-4α; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VSV, vesicular stomatitis virus; VSVpv, VSV pseudotype virus; tet, tetracycline; pol, polymerase; MOI, multiplicity of infection; Dox, doxycycline; IFN, interferon-α8; ES cells, embryonic stem cells.

* Corresponding authors. Address: Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 8199 (M. Kondoh); Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 8185 (H. Mizuguchi).

E-mail addresses: masuo@phs.osaka-u.ac.jp (M. Kondoh), mizuguch@phs.osaka-u.ac.jp (H. Mizuguchi).

[1] These authors contributed equally to this study.

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doi:10.1016/j.bbrc.2011.11.007

Hepatology Research

Hepatology Research 2011; **41**: 258–269 doi: 10.1111/j.1872-034X.2010.00771.x

Original Article

Studies on virus kinetics using infectious fluorescence-tagged hepatitis C virus cell culture

Machi Yamamoto,[1]* Naoya Sakamoto,[1,2]* Tetsuya Nakamura,[1,3] Yasuhiro Itsui,[1,5] Mina Nakagawa,[1,2] Yuki Nishimura-Sakurai,[1] Sei Kakinuma,[1,2] Seishin Azuma,[1] Kiichiro Tsuchiya,[1] Takanobu Kato,[4] Takaji Wakita[4] and Mamoru Watanabe[1]

[1]*Department of Gastroenterology and Hepatology,* [2]*Department for Hepatitis Control,* [3]*Department of Advanced Therapeutics in Gastrointestinal Diseases, Tokyo Medical and Dental University,* [4]*Department of Virology II, National Institute of Infectious Disease, Tokyo, and* [5]*Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan*

Aim: Studies of the complete hepatitis C virus (HCV) life cycle have become possible with the development of a HCV-JFH1 cell culture system.

Methods: In this study, we constructed two fluorescence protein-tagged recombinant JFH1 virus clones, JFH1-EYFP and JFH1-AsRed, as well as two corresponding clones with adaptive mutations, JFH1-EYFP mutant and JFH1-AsRed mutant, that and were as effective as JFH1 in producing infectious virus particles, and investigated their viral infection life cycles.

Results: After infection of the fluorescence-tagged mutant viruses, infected cells increased exponentially. In cells, EYFP or AsRed and NS5A were expressed as a fusion protein and co-localized in core proteins. The rate of the cell–cell spread was dependent on the cell densities with a maximum of $10^{2.5}$/day. Treatment of cells with interferon or a protease inhibitor suppressed expansion of virus-positive cells.

Conclusion: Taken together, these results indicate that fluorescence-tagged HCV is a useful tool to study virus infection life cycles and to assist in the search for novel antiviral compounds.

Key words: AsRed, confocal laser microscopy, HCV-JFH1 cell culture, protease inhibitor, yellow fluorescence protein

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is characterized frequently by chronic inflammation of the liver, leading to decompensated liver cirrhosis and hepatocellular cancers.[1] Interferon (IFN)-α has been the mainstay of HCV therapy.[2] However, the most effective therapy, pegylated IFN plus ribavirin in combination, can eliminate HCV from only half of the patients treated[3,4] and often is accompanied by substantial side-effects.[5,6] These difficulties in eliminating the virus are attributable mostly to the limited treatment options.[7]

Correspondence: Dr Naoya Sakamoto, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Email: nsakamoto.gast@tmd.ac.jp

*M. Y. and N. S. contributed equally to this work.

Received 15 July 2010; revised 21 November 2010; accepted 12 December 2010.

Hepatitis C virus belongs to the family Flaviviridae. The viruses have positive-strand RNA genomes of approximately 10 kb that encode polyproteins of approximately 3000 amino acids. The protein is post-translationally processed by cellular and viral proteases into at least 10 mature proteins. The viral non-structural (NS) proteins accumulate in the endoplasmic reticulum (ER) and they direct genomic replication and viral protein synthesis.[8,9] Studies of the HCV life cycle and the development of new drugs have long been hampered by the lack of cell culture systems. These problems have been greatly overcome by the development of the HCV subgenomic replicon[10] and HCV-JFH1 cell culture[11] systems.

After the development of HCV-JFH1 cell culture, many variations of reporter protein-tagged HCV systems have been described.[12–15] These reporter systems, however, feature poor or absent virus propagation, secretion and re-infection. The C-terminal end of the NS5A region, which has been used for insertion of

Biochemical and Biophysical Research Communications 416 (2011) 119–124

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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[a] Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[b] Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[c] Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
[d] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

ARTICLE INFO

Article history:
Received 31 October 2011
Available online 10 November 2011

Keywords:
Hepatitis C virus
Induced pluripotent stem cell
Hepatocyte
Infection
Replication
Experimental model

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Host targets are alternative targets for the development of anti-HCV drugs. A liver-specific microRNA (miRNA), miR-122, facilitates the replication of the HCV RNA genome in cultured liver cells [6]. Administration of a chemically modified oligonucleotide complementary to miR-122 results in long-lasting suppression of HCV with no appearance of resistant HCV in chimpanzees [7]. Epidermal

Abbreviations: HCV, hepatitis C virus; iPS-Hep cells, human induced pluripotent stem cells-derived hepatocyte-like cells; HCVpv, HCV pseudotype virus; SR-BI, scavenger receptor class B type I; miRNA, microRNA; EGF-R, epidermal growth factor receptor; EphA2, ephrin factor A2; iPS cells, human induced pluripotent stem cells; FCS, fetal calf serum; Ad, adenovirus; HNF-4α, hepatocyte nuclear factor-4α; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VSV, vesicular stomatitis virus; VSVpv, VSV pseudotype virus; tet, tetracycline; pol, polymerase; MOI, multiplicity of infection; Dox, doxycycline; IFN, interferon-α8; ES cells, embryonic stem cells.

* Corresponding authors. Address: Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 8199 (M. Kondoh); Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 8185 (H. Mizuguchi).

E-mail addresses: masuo@phs.osaka-u.ac.jp (M. Kondoh), mizuguch@phs.osaka-u.ac.jp (H. Mizuguchi).

[1] These authors contributed equally to this study.

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.11.007

Nucleic Acids Research Advance Access published February 8, 2011

Nucleic Acids Research, 2011, 1–8
doi:10.1093/nar/gkr047

Adenovirus vector-mediated assay system for hepatitis C virus replication

Takeshi Yoshida[1], Masuo Kondoh[1,*], Manabu Ojima[1], Hiroyuki Mizuguchi[2], Yoshiaki Yamagishi[1], Naoya Sakamoto[3] and Kiyohito Yagi[1,*]

[1]Laboratory of Bio-Functional Molecular Chemistry, [2]Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka and [3]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

Received April 9, 2010; Revised January 17, 2011; Accepted January 18, 2011

ABSTRACT

The efficient delivery of the hepatitis C virus (HCV) RNA subgenomic replicon into cells is useful for basic and pharmaceutical studies. The adenovirus (Ad) vector is a convenient and efficient tool for the transduction of foreign genes into cells *in vitro* and *in vivo*. However, an Ad vector expressing the HCV replicon has never been developed. In the present study, we developed Ad vector containing an RNA polymerase (pol) I-dependent expression cassette and a tetracycline-controllable RNA pol I-dependent expression system. We prepared a hybrid promoter from the tetracycline-responsive element and the RNA pol I promoter. Ad vector particles coding the hybrid promoter-driven HCV replicon could be amplified, and interferon, an inhibitor of HCV replication, reduced HCV replication in cells transduced with the Ad vector coding HCV replicon. This is the first report of the development of an Ad vector-mediated HCV replicon system.

INTRODUCTION

Hepatitis C virus (HCV) is a member of *Flaviviridae* that contains a 9.6-kb positive-sense RNA genome. A total of 170-million people worldwide are infected with HCV, leading to chronic hepatic inflammation, hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (1). Chronic infection with HCV is a major cause of hepatocellular carcinoma (1). Interferon (IFN) therapy is the gold standard method for HCV patients, but it is effective in only 50% of patients and its use has been limited because of severe side effects (2–4). Additional pharmaceutical therapies are needed to overcome HCV. However, the tropism of HCV is limited to chimpanzees and humans, and the mechanism of HCV infection and replication is not fully understood. The HCV genome encodes a polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (5). An HCV subgenome replicon (called HCV replicon in the present study) consisting of a reporter gene and HCV NS genes has allowed various studies of HCV replication and the development of anti-HCV agents (6–8). The delivery of the HCV genome or HCV replicon is a powerful tool for basic and pharmaceutical research, and the transduction of *in vitro* translated HCV RNA genome is often performed by electroporation. However, a convenient and efficient method to transfer the 9.6-kb HCV RNA genome or the 8–9-kb HCV replicon has never been fully developed.

Transcribed RNAs are classified into rRNAs, mRNAs and short RNAs (tRNAs) in mammalian cells. RNA polymerases differ among the transcribed RNA species: RNA polymerase (pol) I for rRNAs, RNA pol II for mRNA and RNA pol III for short RNAs. RNA pol I transcribes RNA without a 5′-cap structure or a 3′-poly-A tail, and a plasmid vector encoding RNA pol I promoter and terminator has been applied to the development of RNA virus-expression system. For instance, influenza viruses, arenavirus and uukuniemi viruses are generated using RNA pol I-driven expression plasmid vectors coding each segment of negative-sense RNA (9–12). Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to a variety of cell types and tissues *in vitro* and *in vivo* in basic research and clinical therapy. Ad vector can be easily prepared, grown to a high titer, and used to efficiently transfer genes into dividing and non-dividing cells. Furthermore, several types of Ad vectors have been developed to expand their tropism and to increase the size of encoded genes (13,14). Ad vector encoding RNA pol I-driven expression of influenza virus

*To whom correspondence should be addressed. Tel: +81 6 6879 8196; Fax: +81 6 6879 8199; Email: masuo@phs.osaka-u.ac.jp
Correspondence may also be addressed to Kiyohito Yagi. Tel/Fax: +81 6 6879 8195; Email: yagi@phs.osaka-u.ac.jp

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Microbiology and Immunology

Microbiol Immunol 2012; **56**: 1–9
doi:10.1111/j.1348-0421.2011.00403.x

REVIEW

In vitro models for analysis of the hepatitis C virus life cycle

Hussein H. Aly[1,2], Kunitada Shimotohno[3], Makoto Hijikata[4] and Tsukasa Seya[1]

[1]Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, [2]Laboratory of Viral Hepatitis and Host Defense, Hokkaido University Creative Research Institution, Kita-22, Nishi-7, Kita-ku, Sapporo 060-8638, [3]Research Institute, Chiba Institute of Technology, Narashino 275-0016, Chiba and [4]Department of Virology, Virus Research Institute, Kyoto University, Kyoto 606-8507, Japan

ABSTRACT

Chronic hepatitis C virus (HCV) infection affects approximately 170 million people worldwide. HCV infection is a major global health problem as it can be complicated with liver cirrhosis and hepatocellular carcinoma. So far, there is no vaccine available and the non-specific, interferon (IFN)-based treatments now in use have significant side-effects and are frequently ineffective, as only approximately 50% of treated patients with genotypes 1 and 4 demonstrate HCV clearance. The lack of suitable *in vitro* and *in vivo* models for the analysis of HCV infection has hampered elucidation of the HCV life cycle and the development of both protective and therapeutic strategies against HCV infection. The present review focuses on the progress made towards the establishment of such models.

Key words hepatitis C virus, HuH-7 cell, knockout mice, type I interferon.

Chronic HCV infection is a major cause of mortality and morbidity throughout the world, infecting approximately 3.1% of the world's population (1). Only a fraction of acutely infected individuals are able to clear the infection spontaneously, whereas approximately 80% of infected individuals develop a chronic infection (2, 3). Patients with chronic HCV are at increased risk for developing liver fibrosis, cirrhosis, and/or hepatocellular carcinoma. Currently, these long-term complications of chronic HCV infection are the leading indication for liver transplantation (4, 5). Because of the high incidence of new infections by blood transfusions in the 1980s before the discovery of the virus, and because morbidity associated with chronic HCV infection generally takes decades to develop, it is expected that the burden of disease in the near future will rise dramatically.

HCV is an enveloped flavivirus, with a positive-stranded RNA genome of approximately 9600 nucleotides. The coding region is flanked by 5′ and 3′ non-coding regions, which are important for the initiation of translation and regulation of genomic duplication, respectively. The coding region itself is composed of a single open reading frame, which encodes a polyprotein precursor of approximately 3000 amino acids. This polyprotein is cleaved by host and viral proteases into structural and NS proteins (Fig. 1). Replication of the HCV genome involves the synthesis of a full-length negative-stranded RNA intermediate, which in turn provides a template for the de novo production of positive-stranded RNA. Both these synthesis steps are mediated by the viral RNA-dependent RNA polymerase NS5B (6–8). NS5B lacks proofreading abilities, and this leads to a high mutation rate and the

Correspondence
Hussein H. Aly, Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638, Japan.
Tel: +81 11 706 5073; fax: +81 11 706 5056; email: ahussein@med.hokudai.ac.jp

Received 8 September 2011; revised 23 October 2011; accepted 3 November 2011.

List of Abbreviations: 3-D, three-dimensional; 3-D/HF, three-dimensional hollow fiber system; bbHCV, blood borne hepatitis C virus; HCV, hepatitis C virus; HPV/E6E7, human papilloma virus E6/E7 genes; IFN, interferon; IFNAR, interferon A receptor; IRES, internal ribosome entry site; ko, knockout; MDA-5, melanoma differentiation associated gene 5; MEF, mouse embryo fibroblasts; mir199, micro RNA 199; NS proteins, non-structural proteins; PPAR, peroxisome proliferator-activated receptor; RFB, radial flow bioreactor; RIG-I, retinoic acid-inducible gene I; TLR, Toll-like receptor; uPA, urokinase plasminogen activator.

Visualization and Measurement of ATP Levels in Living Cells Replicating Hepatitis C Virus Genome RNA

Tomomi Ando[1,2], Hiromi Imamura[3], Ryosuke Suzuki[1], Hideki Aizaki[1], Toshiki Watanabe[2], Takaji Wakita[1], Tetsuro Suzuki[4]*

1 Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, **2** Graduate School of Frontier Sciences, The University of Tokyo, Tokyo, Japan, **3** The Hakubi Center and Graduate School of Biostudies, Kyoto University, Kyoto, Japan, **4** Hamamatsu University School of Medicine, Department of Infectious Diseases, Hamamatsu, Japan

Abstract

Adenosine 5′-triphosphate (ATP) is the primary energy currency of all living organisms and participates in a variety of cellular processes. Although ATP requirements during viral lifecycles have been examined in a number of studies, a method by which ATP production can be monitored in real-time, and by which ATP can be quantified in individual cells and subcellular compartments, is lacking, thereby hindering studies aimed at elucidating the precise mechanisms by which viral replication energized by ATP is controlled. In this study, we investigated the fluctuation and distribution of ATP in cells during RNA replication of the hepatitis C virus (HCV), a member of the *Flaviviridae* family. We demonstrated that cells involved in viral RNA replication actively consumed ATP, thereby reducing cytoplasmic ATP levels. Subsequently, a method to measure ATP levels at putative subcellular sites of HCV RNA replication in living cells was developed by introducing a recently-established Förster resonance energy transfer (FRET)-based ATP indicator, called ATeam, into the NS5A coding region of the HCV replicon. Using this method, we were able to observe the formation of ATP-enriched dot-like structures, which co-localize with non-structural viral proteins, within the cytoplasm of HCV-replicating cells but not in non-replicating cells. The obtained FRET signals allowed us to estimate ATP concentrations within HCV replicating cells as ~5 mM at possible replicating sites and ~1 mM at peripheral sites that did not appear to be involved in HCV replication. In contrast, cytoplasmic ATP levels in non-replicating Huh-7 cells were estimated as ~2 mM. To our knowledge, this is the first study to demonstrate changes in ATP concentration within cells during replication of the HCV genome and increased ATP levels at distinct sites within replicating cells. ATeam may be a powerful tool for the study of energy metabolism during replication of the viral genome.

Citation: Ando T, Imamura H, Suzuki R, Aizaki H, Watanabe T, et al. (2012) Visualization and Measurement of ATP Levels in Living Cells Replicating Hepatitis C Virus Genome RNA. PLoS Pathog 8(3): e1002561. doi:10.1371/journal.ppat.1002561

Editor: Andrea Gamarnik, Fundación Instituto Leloir-CONICET, Argentina

Received August 22, 2011; **Accepted** January 18, 2012; **Published** March 1, 2012

Funding: This work was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science and Technology of Japan. T.A. is a research fellow of the Japan Society for the Promotion of Science. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: tesuzuki@hama-med.ac.jp

Introduction

Adenosine 5′-triphosphate (ATP) is the major energy currency of cells and is involved in a variety of cellular processes, including the virus life cycle, in which ATP-dependent reactions essential for virus multiplication are catalyzed by viral-encoded enzymes or complexes consisting of viral and host-cell proteins [1]. However, the lack of a real-time monitoring system for ATP has hindered studies aimed at elucidating the mechanisms by which cellular processes are controlled through ATP. A method for measuring ATP levels in individual living cells has recently been developed using a genetically-encoded FRET-based indicator for ATP, called ATeam, which employs the epsilon subunit of a bacterial F_0F_1-ATPase [2]. The epsilon subunit has several theoretical advantages for use as an ATP indicator; i) small size (14 kDa), ii) high specific binding to ATP, iii) ATP binding induces a global conformational change and iv) ATP hydrolysis does not occur following binding [3–5]. The affinity of ATeam for ATP can be adjusted by changing various amino acid residues in the ATP-binding domain within the subunit. ATeam has enabled researchers to examine the subcellular compartmentation of ATP as well as time-dependent changes in cellular ATP levels under various physiological conditions. For example, the ATeam-based method has been used to demonstrate that ATP levels within the mitochondrial matrix are lower than those in the cytoplasm and the nucleus [2].

Hepatitis C virus (HCV) infects 2–3% of the world population and is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [6–8]. HCV possesses a positive-strand RNA genome and belongs to the family *Flaviviridae*. A precursor polyprotein of ~3000 amino acids is post- or co-translationally processed by both viral and host proteases into at least ten viral products. The nonstructural (NS) proteins NS3, NS4A, NS4B, NS5A and NS5B are necessary and sufficient for autonomous HCV RNA replication. These proteins form a membrane-associated replication complex (RC), in which NS5B is the RNA-dependent RNA polymerase (RdRp) responsible for copying the RNA genome of the virus during replication [9,10]. NS3, in addition to its protease activity, functions as a viral helicase capable of separating duplex RNA and DNA in reactions fuelled

Association of Gene Expression Involving Innate Immunity and Genetic Variation in Interleukin 28B With Antiviral Response

Yasuhiro Asahina,[1] Kaoru Tsuchiya,[1] Masaru Muraoka,[1,2] Keisuke Tanaka,[1,2] Yuichiro Suzuki,[1,2] Nobuharu Tamaki,[1] Yoshihide Hoshioka,[1] Yutaka Yasui,[1] Tomoji Katoh,[1] Takanori Hosokawa,[1] Ken Ueda,[1] Hiroyuki Nakanishi,[1] Jun Itakura,[1] Yuka Takahashi,[1] Masayuki Kurosaki,[1] Nobuyuki Enomoto,[2] Sayuri Nitta,[3] Naoya Sakamoto,[3] and Namiki Izumi[1]

Innate immunity plays an important role in host antiviral response to hepatitis C viral (HCV) infection. Recently, single nucleotide polymorphisms (SNPs) of *IL28B* and host response to peginterferon α (PEG-IFNα) and ribavirin (RBV) were shown to be strongly associated. We aimed to determine the gene expression involving innate immunity in *IL28B* genotypes and elucidate its relation to response to antiviral treatment. We genotyped *IL28B* SNPs (rs8099917 and rs12979860) in 88 chronic hepatitis C patients treated with PEG-IFNα-2b/RBV and quantified expressions of viral sensors (*RIG-I, MDA5*, and *LGP2*), adaptor molecule (*IPS-1*), related ubiquitin E3-ligase (*RNF125*), modulators (*ISG15* and *USP18*), and *IL28* (*IFNλ*). Both *IL28B* SNPs were 100% identical; 54 patients possessed rs8099917 TT/rs12979860 CC (*IL28B* major patients) and 34 possessed rs8099917 TG/rs12979860 CT (*IL28B* minor patients). Hepatic expressions of viral sensors and modulators in *IL28B* minor patients were significantly up-regulated compared with that in *IL28B* major patients (≈3.3-fold, $P < 0.001$). However, expression of *IPS-1* was significantly lower in *IL28B* minor patients (1.2-fold, $P = 0.028$). Expressions of viral sensors and modulators were significantly higher in nonvirological responders (NVR) than that in others despite stratification by *IL28B* genotype (≈2.6-fold, $P < 0.001$). Multivariate and ROC analyses indicated that higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio were independent factors for NVR. IPS-1 down-regulation in *IL28B* minor patients was confirmed by western blotting, and the extent of IPS-1 protein cleavage was associated with the variable treatment response. *Conclusion:* Gene expression involving innate immunity is strongly associated with *IL28B* genotype and response to PEG-IFNα/RBV. Both *IL28B* minor allele and higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio are independent factors for NVR. (HEPATOLOGY 2012;55:20-29)

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma in many patients.[1] Pegylated interferon α (PEG-IFNα) and ribavirin (RBV) combination therapy has been used to treat chronic hepatitis C (CH-C) to alter the natural course of this disease. However, 20% patients are nonvirological responders (NVR) whose HCV-RNA does not become negative during the 48 weeks of PEG-IFNα/RBV combination therapy.[2] In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near interleukin 28B

Abbreviations: CH-C, chronic hepatitis C; γ-GTP, γ-glutamyl transpeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HMBS, hydroxymethylbilane synthase; IL28, interleukin 28; IPS-1, IFNβ promoter stimulator 1; ISG15, interferon-stimulated gene 15; MDA5, melanoma differentiation associated gene 5; NVR; nonvirological responders; PEG-IFNα, pegylated interferonα; SNP, single nucleotide polymorphism; RIG-I, retinoic acid-inducible gene I; RBV, ribavirin; RNF125, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TVR, transient virological responder; USP18, ubiquitin-specific protease 18; VR, virological responder.

From the [1]Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan; [2]First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan; [3]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan.

Received May 14, 2011; accepted August 16, 2011.

Supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology and the Japanese Ministry of Welfare, Health and Labor. The funding source had no role in the collection, analysis, or interpretation of the data, or in the decision to submit the article for publication.

Cell. Mol. Life Sci.
DOI 10.1007/s00018-012-0940-6

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Let-7b is a novel regulator of hepatitis C virus replication

Ju-Chien Cheng · Yung-Ju Yeh · Ching-Ping Tseng · Sheng-Da Hsu · Yu-Ling Chang · Naoya Sakamoto · Hsien-Da Huang

Received: 29 September 2011 / Revised: 28 January 2012 / Accepted: 9 February 2012

Abstract The non-coding microRNA (miRNA) is involved in the regulation of hepatitis C virus (HCV) infection and offers an alternative target for developing anti-HCV agent. In this study, we aim to identify novel cellular miRNAs that directly target the HCV genome with anti-HCV therapeutic potential. Bioinformatic analyses were performed to unveil liver-abundant miRNAs with predicted target sequences on HCV genome. Various cell-based systems confirmed that let-7b plays a negative role in HCV expression. In particular, let-7b suppressed HCV replicon activity and down-regulated HCV accumulation leading to reduced infectivity of HCVcc. Mutational analysis identified let-7b binding sites at the coding sequences of NS5B and 5′-UTR of HCV genome that were conserved among various HCV genotypes. We further demonstrated that the underlying mechanism for let-7b-mediated suppression of HCV RNA accumulation was not dependent on inhibition of HCV translation. Let-7b and IFNα-2a also elicited a synergistic inhibitory effect on HCV infection. Together, let-7b represents a novel cellular miRNA that targets the HCV genome and elicits anti-HCV activity. This study thereby sheds new insight into understanding the role of host miRNAs in HCV pathogenesis and to developing a potential anti-HCV therapeutic strategy.

Keywords microRNA · Let-7b · HCV

Abbreviations

miRNA	microRNA
HCV	Hepatitis C virus
MRE	MicroRNA responsive element
IFNα-2a	Peginterferon alpha-2a
IFN	Interferon
LF2000	Lipofectamine 2000
DMEM	Dulbecco's modified Eagle's medium
FITC	Fluorescein isothiocyanate
DAPI	4′,6-diamidino-2-phenylindole

Introduction

Hepatitis C virus (HCV) frequently causes chronic infection, leading to hepatic fibrosis and hepatocellular carcinoma [1]. Due to the lack of viral vaccine, the population affected by HCV infection is increased substantially [2]. With the strong side-effects and the moderate successful rate associated with the first-line interferon (IFN)-based

Ju-Chien Cheng and Yung-Ju Yeh contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00018-012-0940-6) contains supplementary material, which is available to authorized users.

J.-C. Cheng (✉) · Y.-J. Yeh · Y.-L. Chang
Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung 404, Taiwan, ROC
e-mail: jccheng@mail.cmu.edu.tw

C.-P. Tseng
Department of Medical Biotechnology and Laboratory Science, Chang Gung University, Taoyuan, Taiwan, ROC

Y.-J. Yeh · S.-D. Hsu · H.-D. Huang (✉)
Institute of Bioinformatics and Systems Biology, National Chiao Tung University, Hsinchu 300, Taiwan, ROC
e-mail: bryan@mail.nctu.edu.tw

N. Sakamoto
Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

H.-D. Huang
Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 300, Taiwan, ROC

Published online: 06 March 2012

JVI
Journals.ASM.org

Novel Cell Culture-Adapted Genotype 2a Hepatitis C Virus Infectious Clone

Tomoko Date,[a] Takanobu Kato,[a] Junko Kato,[b] Hitoshi Takahashi,[a]* Kenichi Morikawa,[a,c]* Daisuke Akazawa,[a,d] Asako Murayama,[a] Keiko Tanaka-Kaneko,[e] Tetsutaro Sata,[e]* Yasuhito Tanaka,[f] Masashi Mizokami,[g] and Takaji Wakita[a]

Department of Virology II, National Institute of Infectious Diseases, Tokyo,[a] Institute of Geriatrics, Tokyo Women's Medical University, Tokyo,[b] Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Tokyo,[c] Pharmaceutical Research Laboratories, Toray Industries, Inc., Kanagawa,[d] Department of Pathology, National Institute of Infectious Diseases, Tokyo,[e] Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya,[f] and The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Chiba,[g] Japan

Although the recently developed infectious hepatitis C virus system that uses the JFH-1 clone enables the study of whole HCV viral life cycles, limited particular HCV strains have been available with the system. In this study, we isolated another genotype 2a HCV cDNA, the JFH-2 strain, from a patient with fulminant hepatitis. JFH-2 subgenomic replicons were constructed. HuH-7 cells transfected with *in vitro* transcribed replicon RNAs were cultured with G418, and selected colonies were isolated and expanded. From sequencing analysis of the replicon genome, several mutations were found. Some of the mutations enhanced JFH-2 replication; the 2217AS mutation in the NS5A interferon sensitivity-determining region exhibited the strongest adaptive effect. Interestingly, a full-length chimeric or wild-type JFH-2 genome with the adaptive mutation could replicate in Huh-7.5.1 cells and produce infectious virus after extensive passages of the virus genome-replicating cells. Virus infection efficiency was sufficient for autonomous virus propagation in cultured cells. Additional mutations were identified in the infectious virus genome. Interestingly, full-length viral RNA synthesized from the cDNA clone with these adaptive mutations was infectious for cultured cells. This approach may be applicable for the establishment of new infectious HCV clones.

Hepatitis C virus (HCV) is a principal agent in posttransfusion and sporadic acute hepatitis (6, 19). HCV belongs to the *Flaviviridae* family and *Hepacivirus* genus. Infection with HCV leads to chronic liver diseases, including cirrhosis and hepatocellular carcinoma (16). HCV is a major public health problem, infecting an estimated 170 million people worldwide (6, 16, 19). Current standard therapy for HCV-related chronic hepatitis is based on the combination of interferon (IFN) and ribavirin although virus eradication rates are limited to around 50% (7, 24, 30). Telaprevir and boceprevir were approved by the U.S. Food and Drug Administration in 2011 in combination with pegylated alpha interferon and ribavirin for the treatment of genotype 1 chronic hepatitis C (34, 35). Both agents inhibit the NS3-NS4A serine protease essential for replication of HCV (25, 36). It is important to develop more anti-HCV drugs with different modes of action to achieve greater efficacy and to avoid the emergence of drug-resistant viruses. To that end, a detailed understanding of the viral replication mechanism is needed to discover novel antiviral targets. An efficient virus culture system is indispensable for detailed analysis of HCV life cycles. In an important development, a subgenomic HCV RNA replicon system has been developed (22) to assess HCV replication in cultured cells. Furthermore, an efficient HCV culture system was established by using a JFH-1 strain virus isolated from a fulminant hepatitis patient (20, 38, 41). By transfection of *in vitro* transcribed full-length JFH-1 HCV RNA into HuH-7 cells, efficient JFH-1 RNA replication and infectious viral particle production were detected. However, this efficient virus production was not reproduced by other HCV strains, even when adaptive mutations were introduced to enhance the replication efficiency in cultured cells (29). Thus, other HCV strains that can replicate in cultured cells and produce infectious virus particles are needed. The J6CF strain is infectious to chimpanzees but does not replicate in cultured cells (26, 27, 40). We constructed chimeric replicon and virus constructs of the J6CF and JFH-1 strains to elucidate the difference in their molecular mechanisms (26, 27). We determined that the NS3 helicase and the NS5B to 3′X regions are important for the efficient replication of the JFH-1 strain and that several amino acid mutations in the C terminus of NS5B are pivotal for replication. However, we could not rescue the replication of other virus strains, such as Con1, with these mutations. This result indicates that different approaches are needed to create replication-competent virus strains in cultured cells.

In the present study, we isolated HCV cDNA, named JFH-2, from a fulminant hepatitis patient. The replication efficiency of the JFH-2 clone in the subgenomic replicon assay was lower than that of JFH-1 although the introduction of adaptive mutations enhanced JFH-2 replication. Interestingly, the full-length chimeric or wild-type JFH-2 genome with adaptive mutations could replicate and produce infectious virus particles. The virus infection efficiency was sufficient for autonomous virus propagation in cultured cells.

MATERIALS AND METHODS

Cell culture system. HuH-7, Huh-7.5.1 (a generous gift from Francis V. Chisari), and Huh7-25 cells were cultured in 5% CO_2 at 37°C in Dulbec-

Received 29 December 2011 **Accepted** 2 July 2012
Published ahead of print 11 July 2012
Address correspondence to Takaji Wakita, wakita@nih.go.jp.
* Present address: Hitoshi Takahashi, Influenza Virus Research Center, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan; Kenichi Morikawa, Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Lausanne, Switzerland; Tetsutaro Sata, Toyama Institute of Health, Toyama, Japan.

doi:10.1128/JVI.07235-11

aptara | MIM | mim'437 | Dispatch: March 16, 2012 | CE: N/A
Journal | MSP No. | No. of pages: 10 | PE: Helen

Microbiol Immunol 2012; **00**: 1–10
doi:10.1111/j.1348-0421.2012.00437.x

ORIGINAL ARTICLE

Replication and infectivity of a novel genotype 1b hepatitis C virus clone

Tomoko Date[1], Kenichi Morikawa[1,2,†], Yasuhito Tanaka[3], Keiko Tanaka-Kaneko[4], Tetsutaro Sata[4,‡], Masashi Mizokami[5] and Takaji Wakita[1]

Departments of [1]Virology II and [4]Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, [2]Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, [3]Department of Virology & Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, and [5]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1 Kounodai, Ichikawa 272-8516, Japan

ABSTRACT

Hepatitis C virus infection is a major public health problem because of an estimated 170 million carriers worldwide. Genotype 1b is the major subtype of HCV in many countries and is resistant to interferon therapy. Study of the viral life cycle is important for understanding the mechanisms of interferon resistance of genotype 1b HCV strains. For such studies, genotype 1b HCV strains that can replicate and produce infectious virus particles in cultured cells are required. In the present study, we isolated HCV cDNA, which we named the NC1 strain, from a patient with acute severe hepatitis. Subgenomic replicon experiments revealed that several mutations enhanced the colony-formation efficiency of the NC1 replicon. The full-length NC1 genome with these adaptive mutations could replicate in cultured cells and produce infectious virus particles. The density gradient profile and morphology of the secreted virus particles were similar to those reported for the JFH-1 virus. Further introduction of a combination of mutations of the NS3 and NS5a regions into the NC1 mutants further enhanced secreted core protein levels and infectious virus titers in the culture medium of HCV-RNA-transfected cells. However, the virus infection efficiency was not sufficient for autonomous virus propagation in cultured cells. In conclusion, we established a novel cell culture-adapted genotype 1b HCV strain, termed NC1, which can produce infectious virus when the viral RNA is transfected into cells. This system provides an important opportunity for studying the life cycle of the genotype 1b HCV.

Key words genotype 1b, hepatitis C virus (HCV), replicon, virus culture.

Hepatitis C virus infection leads to chronic liver diseases including cirrhosis and hepatocellular carcinoma, and is a major public health problem because of an estimated 170 million carriers worldwide (1–3). HCV is a plus-strand RNA virus that displays marked genetic heterogeneity and is currently classified into six major genotypes (4). Some HCV genotypes display regional distribution, although genotypes 1 and 2 occur worldwide. Genotype 1b is the major subtype in Japan, whereas genotype 2a is the most common minor subtype (5). Infection with genotype 1b HCV is known to be resistant to interferon therapy, whereas infection with genotype 2a is

Correspondence
Takaji Wakita, Second Department of Virology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel: +81 3 5285 1111; fax: +81 3-5285 1161; email: wakita@nih.go.jp
Present addresses
[†]Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Lausanne, Switzerland.
[‡]Toyama Institute of Health, Toyama, Japan.

Received 19 January 2012; revised 23 January 2012; accepted 26 January 2012.

List of Abbreviations: CFU, colony-forming units; DMEM, Dulbecco's modified Eagle's medium; EMCV, encephalomyocarditis virus; ffu, focus-forming units; HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosomal entry site; PI, protease inhibitors; RTD-PCR, real-time detection RT-PCR.

PLOS ONE

Inhibition of Both Protease and Helicase Activities of Hepatitis C Virus NS3 by an Ethyl Acetate Extract of Marine Sponge *Amphimedon* sp.

Yuusuke Fujimoto[1], Kazi Abdus Salam[2☯], Atsushi Furuta[3,4☯], Yasuyoshi Matsuda[3,4], Osamu Fujita[3,4], Hidenori Tani[5], Masanori Ikeda[6], Nobuyuki Kato[6], Naoya Sakamoto[7], Shinya Maekawa[8], Nobuyuki Enomoto[8], Nicole J. de Voogd[9], Masamichi Nakakoshi[10], Masayoshi Tsubuki[10], Yuji Sekiguchi[3], Satoshi Tsuneda[4], Nobuyoshi Akimitsu[2], Naohiro Noda[3,4], Atsuya Yamashita[1]*, Junichi Tanaka[11]*, Kohji Moriishi[1]*

1 Department of Microbiology, Division of Medicine, Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan, **2** Radioisotope Center, The University of Tokyo, Tokyo, Japan, **3** Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan, **4** Department of Life Science and Medical Bioscience, Waseda University, Tokyo, Japan, **5** Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan, **6** Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, **7** Department of Gastroenterology and Hepatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan, **8** First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan, **9** Netherlands Centre for Biodiversity Naturalis, Leiden, The Netherlands, **10** Institute of Medical Chemistry, Hoshi University, Tokyo, Japan, **11** Department of Chemistry, Biology and Marine Science, University of the Ryukyus, Okinawa, Japan

Abstract

Combination therapy with ribavirin, interferon, and viral protease inhibitors could be expected to elicit a high level of sustained virologic response in patients infected with hepatitis C virus (HCV). However, several severe side effects of this combination therapy have been encountered in clinical trials. In order to develop more effective and safer anti-HCV compounds, we employed the replicon systems derived from several strains of HCV to screen 84 extracts from 54 organisms that were gathered from the sea surrounding Okinawa Prefecture, Japan. The ethyl acetate-soluble extract that was prepared from marine sponge *Amphimedon* sp. showed the highest inhibitory effect on viral replication, with EC_{50} values of 1.5 and 24.9 μg/ml in sub-genomic replicon cell lines derived from genotypes 1b and 2a, respectively. But the extract had no effect on interferon-inducing signaling or cytotoxicity. Treatment with the extract inhibited virus production by 30% relative to the control in the JFH1-Huh7 cell culture system. The *in vitro* enzymological assays revealed that treatment with the extract suppressed both helicase and protease activities of NS3 with IC_{50} values of 18.9 and 10.9 μg/ml, respectively. Treatment with the extract of *Amphimedon* sp. inhibited RNA-binding ability but not ATPase activity. These results suggest that the novel compound(s) included in *Amphimedon* sp. can target the protease and helicase activities of HCV NS3.

Citation: Fujimoto Y, Salam KA, Furuta A, Matsuda Y, Fujita O, et al. (2012) Inhibition of Both Protease and Helicase Activities of Hepatitis C Virus NS3 by an Ethyl Acetate Extract of Marine Sponge *Amphimedon* sp.. PLoS ONE 7(11): e48685. doi:10.1371/journal.pone.0048685

Editor: Tetsuo Takehara, Osaka University Graduate School of Medicine, Japan

Received June 16, 2012; **Accepted** October 1, 2012; **Published** November 7, 2012

Funding: This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare (http://www.mhlw.go.jp/) and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (http://www.mext.go.jp/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: atsuyay@yamanashi.ac.jp (AY); jtanaka@sci.u-ryukyu.ac.jp (JT); kmoriishi@yamanashi.ac.jp (KM)

☯ These authors contributed equally to this work.

Introduction

Hepatitis C virus (HCV) is an enveloped RNA virus of the genus *Hepacivirus* of the *Flaviviridae* family. More than 170 million patients persistently infected with HCV have been reported worldwide, leading to liver diseases including steatosis, cirrhosis, and hepatocellular carcinoma [1,2]. The genome of HCV is characterized as a single positive-strand RNA with a nucleotide length of 9.6 kb, flanked by 5′ and 3′-untranslated regions (UTRs). The genomic RNA encodes a large polyprotein consisting of approximately 3,000 amino acids [3], which is translated under the control of an internal ribosome entry site (IRES) located within the 5′-UTR of the genomic RNA [4]. The translated polyprotein is cleaved by host and viral proteases, resulting in 10 mature viral proteins [3]. The structural proteins, consisting of core, E1, and E2, are located in the N-terminal quarter of the polyprotein, followed by viroporin p7, which has not yet been classified into a structural or nonstructural protein. Further cleavage of the remaining portion by viral proteases produces six nonstructural proteins–NS2, NS3, NS4A, NS4B, NS5A, and NS5B–which form a viral replication complex with various host factors. The viral protease NS2 cleaves its own C-terminal between NS2 and NS3. After that, NS3 cleaves the C-terminal ends of NS3 and NS4A and then forms a complex with NS4A. The NS3/4A complex becomes a fully active form to cleave the C-terminal parts of the polyprotein, including nonstructural proteins. NS3 also possesses

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ENT1, a Ribavirin Transporter, Plays a Pivotal Role in Antiviral Efficacy of Ribavirin in a Hepatitis C Virus Replication Cell System

Minami Iikura,[a] Tomomi Furihata,[a] Misa Mizuguchi,[a] Miki Nagai,[a] Masanori Ikeda,[b] Nobuyuki Kato,[b] Akihito Tsubota,[c] and Kan Chiba[a]

Laboratory of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan[a]; Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan[b]; and Institute of Clinical Medicine and Research, Jikei University School of Medicine, Chiba, Japan[c]

We previously showed that equilibrative nucleoside transporter 1 (ENT1) is a primary ribavirin transporter in human hepatocytes. However, because the role of this transporter in the antiviral mechanism of the drug remains unclear, the present study aimed to elucidate the role of ENT1 in ribavirin antiviral action. OR6 cells, a hepatitis C virus (HCV) replication system, were used to evaluate both ribavirin uptake and efficacy. The ribavirin transporter in OR6 cells was identified by mRNA expression analyses and transport assays. Nitrobenzylmercaptopurine riboside (NBMPR) and micro-RNA targeted to ENT1 mRNA (miR-ENT1) were used to reduce the ribavirin uptake level in OR6 cells. Our results showed that ribavirin antiviral activity was associated with its accumulation in OR6 cells, which was also closely associated with the uptake of the drug. It was found that the primary ribavirin transporter in OR6 cells was ENT1 and that inhibition of ENT1-mediated ribavirin uptake by NBMPR significantly attenuated the antiviral activity of the drug as well as its accumulation in OR6 cells. The results also showed that even a small reduction in the ENT1-mediated ribavirin uptake, achieved in this case using miR-ENT1, caused a significant decrease in its antiviral activity, thus indicating that the ENT1-mediated ribavirin uptake level determined its antiviral activity level in OR6 cells. In conclusion, our results show that by facilitating its uptake and accumulation in OR6 cells, ENT1 plays a pivotal role in the antiviral effectiveness of ribavirin and therefore provides an important insight into the efficacy of the drug in anti-HCV therapy.

Chronic hepatitis C is a major cause of liver cirrhosis and hepatocellular carcinoma, and a combination of interferon-α (IFN-α) and ribavirin is a standard anti-hepatitis C virus (HCV) therapy. Since the addition of ribavirin to IFN-α significantly improves the rate of sustained virologic response (SVR) (40 to 60% in genotype 1 patients) (5), the drug plays a key role in current anti-HCV therapy.

Ribavirin, a purine nucleoside analog, is phosphorylated intracellularly to form mono-, di-, and tri-phosphates, which then accumulate within cells at high concentrations (4, 13). While the primary anti-HCV mechanisms of the drug are still under debate, it is considered likely that the important actions take place within the cells themselves, and several mechanisms have been proposed to explain what occurs there. These include inhibition of inosine monophosphate dehydrogenase (reviewed in references 4 and 7 and references therein). Additionally, a recent study revealed that ribavirin potentiates IFN-α action by augmenting IFN-stimulated induction of gene expression (16).

Taking into consideration the above-mentioned mechanisms, it is reasonable to assume that the uptake of ribavirin into hepatocytes is a prerequisite for its antiviral activity. Since ribavirin is a hydrophilic molecule, import of the drug into cells requires host nucleoside transporters, which are divided into two families: equilibrative nucleoside transporters (such as ENT1 to ENT4) and concentrative nucleoside transporters (such as CNT1 to CNT3) (9). ENTs are facilitated transporters, while CNTs are sodium-dependent active transporters. These transporters differ in tissue distribution, substrate preference, and inhibitor sensitivity. For example, sensitivities to inhibition by nitrobenzylmercaptopurine riboside (NBMPR) are different between ENT1 and ENT2 (20).

Our recent investigations into the ribavirin uptake system in human hepatocytes determined that ENT1 is a primary ribavirin uptake transporter (6). In addition, Morello et al. (12) reported the association of an intronic single nucleotide polymorphism (SNP) of the *SLC29A1* (ENT1) gene with rapid virologic response (RVR; defined as an undetectable serum HCV RNA level at week 4) of treatment of genotype-1 Caucasian patients. More recently, Tsubota and colleagues revealed that another intronic SNP in the *SLC29A1* gene is associated with SVR, as well as RVR, in genotype-1 Japanese patients (18). Based on these findings, it can be hypothesized that ENT1 plays an essential role in ribavirin anti-HCV activity.

In the present study, along with a detailed characterization of ribavirin uptake and its relationship to antiviral activity, we tested the above-mentioned hypothesis through the use of OR6 cells, which have been established as an efficient replication system for the HCV RNA genome. The HCV replication level was evaluated by monitoring the level of *Renilla* luciferase activity (8), which enabled us to simultaneously evaluate both ribavirin uptake and its antiviral activity.

MATERIALS AND METHODS

Cell culture. OR6 cells were cloned from ORN/C-5B/KE cells (derived from Huh-7 cells) supporting genome-length HCV RNA (strain O of

Received 20 September 2011 Returned for modification 24 October 2011
Accepted 27 December 2011
Published ahead of print 9 January 2012
Address correspondence to Tomomi Furihata, tomomif@faculty.chiba-u.jp.
Supplemental material for this article may be found at http://aac.asm.org/.

doi:10.1128/AAC.05762-11

J Gastroenterol
DOI 10.1007/s00535-012-0579-y

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Anemia and thrombocytosis induced by ribavirin monotherapy in patients with chronic hepatitis C

Tomoe Kobayashi · Shuhei Hige · Katsumi Terashita · Masato Nakai · Hiromasa Horimoto · Takuya Sho · Mitsuru Nakanishi · Koji Ogawa · Makoto Chuma · Naoya Sakamoto · Masahiro Asaka

Received: 21 December 2011 / Accepted: 28 February 2012

Abstract
Background An inosine triphosphatase (*ITPA*) single-nucleotide polymorphism (SNP) is associated with anemia induced by pegylated interferon and ribavirin (RBV) combination therapy in patients with chronic hepatitis C (CHC). However, there are very few reports on the hematological effects of RBV monotherapy. Here, hematological changes were monitored in patients with CHC who received RBV monotherapy, and the mechanism of these changes was investigated.
Methods Patients with CHC ($n = 30$) received RBV monotherapy for 4 weeks. The RBV dose was determined on the basis of body weight. Complete blood count, and serum erythropoietin (EPO) and thrombopoietin (TPO) levels were assessed. The associations between these parameters and the *ITPA* SNP (*rs1127354*) were analyzed.
Results Over the 4 weeks, the median hemoglobin level of all patients decreased significantly, from 13.6 (10.5–16.6) to 11.7 (9.4–14.9) g/dl ($P < 0.001$), and the platelet counts increased, from 14.0×10^4 ($8.9–37.4 \times 10^4$) to 15.8×10^4 ($10.2–40.6 \times 10^4$) /mm^3 ($P = 0.003$). At week 4, hemoglobin levels differed between patients with the *ITPA* CC genotype and those with the AA or AC genotypes [11.1 (9.4–13.5) vs. 12.9 (12.5–14.9) g/dl, $P = 0.001$]. The platelet change ratio (i.e., platelet count at week 4/platelet count at baseline) in the patients with developing anemia was correlated with the increase in the serum EPO level over 4 weeks ($r = 0.88$, $P = 0.002$), but not with the increase in the serum TPO level over 4 weeks.
Conclusions RBV monotherapy induced anemia and affected thrombocytosis in patients with CHC. Elevated endogenous EPO may stimulate platelet production.

Keywords Ribavirin · Anemia · Erythropoietin · Thrombocytosis · *ITPA* SNP

Abbreviations

ITPA	Inosine triphosphatase
SNP	Single-nucleotide polymorphism
PEG-IFN	Pegylated interferon
RBV	Ribavirin
CHC	Chronic hepatitis C
EPO	Erythropoietin
TPO	Thrombopoietin
HCV	Hepatitis C virus
GWASs	Genome-wide association studies
IL28B	Interleukin 28B
DDRGK1	DDRGK domain-containing protein 1

T. Kobayashi · S. Hige (✉) · K. Terashita · M. Nakai · H. Horimoto · T. Sho · M. Nakanishi · M. Chuma
Department of Gastroenterology,
Hokkaido University Hospital, North 17 Jo,
West 5 Cho-me, Kita-ku, Sapporo 060-8638, Japan
e-mail: shuhei-h@med.hokudai.ac.jp

T. Kobayashi
e-mail: tk990063@med.hokudai.ac.jp

K. Ogawa
Department of Gastroenterology, Hakodate Municipal Hospital,
10-1 Minato-cho, Hakodate 041-8680, Japan

N. Sakamoto
Department of Gastroenterology and Hepatology,
Tokyo Medical and Dental University, 1-5-45 Yushima,
Bunkyo-ku, Tokyo 113-8519, Japan

M. Asaka
Department of Cancer Preventive Medicine, Hokkaido University Graduate School of Medicine, North 12 Jo,
West 7 Cho-me, Kita-ku, Sapporo 060-0812, Japan

Published online: 30 March 2012

Upregulation of nuclear PA28γ expression in cirrhosis and hepatocellular carcinoma

MOTOI KONDO[1], KOHJI MORIISHI[2], HIROSHI WADA[3], TAKEHIRO NODA[3], SHIGERU MARUBASHI[3], KENICHI WAKASA[4], YOSHIHARU MATSUURA[2], YUICHIRO DOKI[3], MASAKI MORI[3] and HIROAKI NAGANO[3]

[1]Evidence Based Medical Research Center, Osaka; [2]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka; [3]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka; [4]Department of Diagnostic Pathology, Graduate School of Medicine, Osaka City University, Osaka, Japan

Received September 16, 2011; Accepted December 2, 2011

DOI: 10.3892/etm.2011.415

Abstract. We previously reported that proteasome activator 28γ (PA28γ) is an oncogenic protein in hepatitis C virus (HCV) core protein transgenic mice. The aim of this study was to determine the role of PA28γ expression at the protein level in the development and progression of human hepatocarcinogenesis and hepatocellular carcinoma (HCC). Samples from tissues representing a wide spectrum of liver disease were analyzed, including histologically normal livers (n=5), HCV-related chronic hepatitis (CH) (n=15) and cirrhosis (n=31). The level of nuclear PA28γ increased with the progression of liver disease from CH to cirrhosis. The majority of cirrhotic livers (68%; 21/31) displayed high nuclear PA28γ expression. However, in half of the HCCs (50%; 18/36), little or no nuclear PA28γ expression was observed, while the remaining 50% (18/36) of the cases displayed high levels of nuclear PA28γ expression. A clinicopathological survey demonstrated a significant correlation between nuclear PA28γ expression and capsular invasion in HCC (P=0.026); a striking difference was found between nuclear PA28γ expression in non-tumor tissues and shorter disease-free survival (P<0.01). Moreover, nuclear PA28γ expression in non-tumor tissues correlated with the expression of molecules related to the genesis of hepatic steatosis and HCC, such as sterol regulatory element binding protein-1c mRNA. The findings suggest the involvement of nuclear PA28γ expression in the progression and relapse of HCC, and suggest that nuclear PA28γ is a potentially suitable target for the prevention and/or treatment of HCC.

Correspondence to: Dr Hiroaki Nagano, Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka E-2, Suita, Osaka 565-0871, Japan
E-mail: hnagano@gesurg.med.osaka-u.ac.jp

Abbreviations: CH, chronic hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PA, proteasome activator; PBGD, porphobilinogen deaminase; RT-PCR, reverse transcription-polymerase chain reaction

Key words: proteasome activator 28γ, hepatocellular carcinoma, cirrhosis, western blotting, immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for approximately 6% of all human carcinomas and 1 million deaths annually, with an estimated number of new cases of over 500,000/year (1). Clinical and experimental evidence suggests a link between infection with hepatitis C virus (HCV) and/or hepatitis B virus (HBV), chronic hepatitis (CH) and cirrhosis, as well as the progression of HCC. Liver cirrhosis is observed in up to 90% of patients with HCC, and HCV is the causative factor in 80% and HBV in 10% of cases in Japan (2-5). In the United States, almost 4 million individuals are infected with HCV each year which progresses to chronic hepatitis C, which could potentially progress to liver cirrhosis. The results are often liver failure or HCC. Chronic hepatitis C is the nation's leading cause of HCC, and according to the American Liver Foundation, is also the leading reason for liver transplantation. In Japan, HCV and/or HBV-based hepatitis and cirrhosis are also serious problems since they progress to HCC at a ratio of 5 to 7% per year (4,5). These findings strongly suggest the existence of a link between hepatocarcinogenesis and HCV/HBV infection and chronic liver inflammation.

Various therapies are currently in use for HCC. These include surgical resection, percutaneous ethanol injection (PEI), systemic or arterial chemotherapy using either single or combination drugs, transcatheter arterial chemoembolization (TACE), hormonal therapy and selective radiotherapy. However, the prognosis of patients with HCC remains poor, as they often develop intrahepatic and/or multicentric tumor recurrence, at a rate of 20-40% within 1 year, and ~80% within 5 years of therapy even when curative treatment is applied (6-9). Liver transplantation offers the best prognosis for patients with small HCC, although its use is limited due to the scarcity of donor organs. Therefore, an effective therapeutic strategy against HCC is required.

Research Article

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Data mining model using simple and readily available factors could identify patients at high risk for hepatocellular carcinoma in chronic hepatitis C

Masayuki Kurosaki[1], Naoki Hiramatsu[2], Minoru Sakamoto[3], Yoshiyuki Suzuki[4], Manabu Iwasaki[5], Akihiro Tamori[6], Kentaro Matsuura[7], Sei Kakinuma[8], Fuminaka Sugauchi[9], Naoya Sakamoto[8], Mina Nakagawa[8], Namiki Izumi[1,*]

[1]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan;* [2]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan;* [3]*First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan;* [4]*Department of Hepatology, Toranomon Hospital, Tokyo, Japan;* [5]*Department of Computer and Information Science, Seikei University, Tokyo, Japan;* [6]*Department of Hepatology, Osaka City University Medical School, Osaka, Japan;* [7]*Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan;* [8]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan;* [9]*Department of Gastroenterology, Nagoya Koseiin Medical Welfare Center, Nagoya, Japan*

Background & Aims: Assessment of the risk of hepatocellular carcinoma (HCC) development is essential for formulating personalized surveillance or antiviral treatment plan for chronic hepatitis C. We aimed to build a simple model for the identification of patients at high risk of developing HCC.

Methods: Chronic hepatitis C patients followed for at least 5 years (n = 1003) were analyzed by data mining to build a predictive model for HCC development. The model was externally validated using a cohort of 1072 patients (472 with sustained virological response (SVR) and 600 with nonSVR to PEG-interferon plus ribavirin therapy).

Results: On the basis of factors such as age, platelet, albumin, and aspartate aminotransferase, the HCC risk prediction model identified subgroups with high-, intermediate-, and low-risk of HCC with a 5-year HCC development rate of 20.9%, 6.3–7.3%, and 0–1.5%, respectively. The reproducibility of the model was confirmed through external validation ($r^2 = 0.981$). The 10-year HCC development rate was also significantly higher in the high- and intermediate-risk group than in the low-risk group (24.5% *vs.* 4.8%; $p < 0.0001$). In the high-and intermediate-risk group, the incidence of HCC development was significantly reduced in patients with SVR compared to those with nonSVR (5-year rate, 9.5% *vs.* 4.5%; $p = 0.040$).

Conclusions: The HCC risk prediction model uses simple and readily available factors and identifies patients at a high risk of HCC development. The model allows physicians to identify patients requiring HCC surveillance and those who benefit from IFN therapy to prevent HCC.

Keywords: Decision tree; Prediction; Pegylated interferon; Ribavirin; Risk.
Received 27 May 2011; received in revised form 8 August 2011; accepted 4 September 2011; available online 23 October 2011

* Corresponding author. Address: Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan. Tel.: +81 422 32 3111; fax: +81 422 32 9551.
E-mail address: nizumi@musashino.jrc.or.jp (N. Izumi).

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide [1] and its incidence is increasing in many countries [2]. Chronic viral hepatitis is responsible for 80% of all HCC cases [2]. The need to conduct HCC surveillance should be determined according to the risk of HCC development because this surveillance is cost-effective only in populations with an annualized cancer development rate of $\geqslant 1.5\%$ [3]. The annualized rate of developing HCC from type C liver cirrhosis is 2–8% [4–6], indicating that this population with type C liver cirrhosis needs surveillance. However, the annualized rate of HCC development is <1.5% in patients with chronic hepatitis C but without cirrhosis and the benefit of surveillance for all patients with chronic hepatitis has not yet been established [3]. HCC surveillance may be needed for patients with advanced fibrosis because the risk of HCC development increases in parallel with the progression of liver fibrosis [7,8]. Liver biopsy is the most accurate means of diagnosing fibrosis, but a single liver biopsy cannot indicate long-term prognosis because liver fibrosis progresses over time. Serial liver biopsies are not feasible because of the procedure's invasiveness. Moreover, factors other than fibrosis, such as advanced age, obesity, sex, lower albumin, and low platelet counts, also contribute to the development of HCC from chronic hepatitis C [8–11]. Therefore, these factors must be considered while assessing the risk of HCC development.

A meta-analysis of controlled trials [12] has shown that interferon (IFN) therapy reduced the rate of HCC development in patients with type C liver cirrhosis. However, there was a marked heterogeneity in the magnitude of the prevention effect

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Journal of Hepatology **2012** vol. 56 | 602–608

Antiviral Therapy 2012; 17:35-43 (doi: 10.3851/IMP1923)

Original article

Age and total ribavirin dose are independent predictors of relapse after interferon therapy in chronic hepatitis C revealed by data mining analysis

*Masayuki Kurosaki[1], Naoki Hiramatsu[2], Minoru Sakamoto[3], Yoshiyuki Suzuki[4], Manabu Iwasaki[5], Akihiro Tamori[6], Kentaro Matsuura[7], Sei Kakinuma[8], Fuminaka Sugauchi[9], Naoya Sakamoto[8], Mina Nakagawa[8], Hiroshi Yatsuhashi[10], Namiki Izumi[1]**

[1]Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan
[2]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan
[3]First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan
[4]Department of Hepatology, Toranomon Hospital, Tokyo, Japan
[5]Department of Computer and Information Science, Seikei University, Tokyo, Japan
[6]Department of Hepatology, Osaka City University Medical School, Osaka, Japan
[7]Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[8]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan
[9]Department of Gastroenterology, Nagoya Koseiin Medical Welfare Center, Nagoya, Japan
[10]Clinical Research Center, National Nagasaki Medical Center, Nagasaki, Japan

*Corresponding author e-mail: nizumi@musashino.jrc.or.jp

Background: This study aimed to define factors associated with relapse among responders to pegylated interferon (PEG-IFN) plus ribavirin (RBV) therapy in chronic hepatitis C.

Methods: A cohort of genotype 1b chronic hepatitis C patients treated with PEG-IFN plus RBV and who had an undetectable HCV RNA by week 12 (n=951) were randomly assigned to model derivation (n=636) or internal validation (n=315) groups. An independent cohort (n=598) were used for an external validation. A decision tree model for relapse was explored using data mining analysis.

Results: The data mining analysis defined five subgroups of patients with variable rates of relapse ranging from 13% to 52%. The reproducibility of the model was confirmed by internal and external validations (r^2=0.79 and 0.83, respectively). Patients with undetectable HCV RNA at week 4 had the lowest risk of relapse (13%), followed by patients <60 years with undetectable HCV RNA at week 5–12 who received ≥3.0 g/kg of body weight of RBV (16%). Older patients with a total RBV dose <3.0 g/kg had the highest risk of relapse (52%). Higher RBV dose beyond 3.0 g/kg was associated with further decrease of relapse rate among patients <60 years (up to 11%) but not among older patients whose relapse rate remained stable around 30%.

Conclusions: Data mining analysis revealed that time to HCV RNA negativity, age and total RBV dose was associated with relapse. To prevent relapse, ≥3.0 g/kg of RBV should be administered. Higher dose of RBV may be beneficial in patients <60 years.

Introduction

The currently recommended therapy for chronic hepatitis C is a combination of pegylated interferon (PEG-IFN) plus ribavirin (RBV) [1]. This therapy is effective in 50% of patients with HCV genotype 1b [2,3]. The most reliable predictor of sustained virological response (SVR) is the response during early weeks of therapy. A satisfactory response to therapy in the early weeks is associated with a high rate of SVR [4–8]. A basic concept of response-guided therapy is to modify the duration of therapy according to the time to HCV RNA negativity. Extended therapy may be given to patients with delayed virological response [9–13]. Modification of duration of therapy or drug dose may also be necessary in patients with early virological

American Journal of Transplantation 2012; 12: 2348–2362
Wiley Periodicals Inc.

doi: 10.1111/j.1600-6143.2012.04116.x

Cyclophilin A and Nuclear Factor of Activated T Cells Are Essential in Cyclosporine-Mediated Suppression of Polyomavirus BK Replication

Y. J. Li[a,b], H. H. Wu[a,b], C. H. Weng[a,b], Y. C. Chen[a], C. C. Hung[a], C. W. Yang[a], R. Y. L. Wang[c], N. Sakamoto[d] and Y. C. Tian[a,*]

[a] *Kidney Research Center, Department of Nephrology, Chang Gung Memorial Hospital, Taipei, Taiwan and Chang Gung University, Tao Yuan, Taiwan*
[b] *Graduate Institute of Clinical Medical Sciences, Chang Gung University, Taiwan*
[c] *Department of Biomedical Sciences, Chang Gung University, TaoYuan, Taiwan*
[d] *Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan*
**Corresponding author: Ya-Chung Tian, dryctian@adm.cgmh.org.tw*

[Correction made after online publication May 29, 2012: author affiliations have been updated.]

Immunosuppressants have impacts on the development of polyomavirus-associated nephropathy. We previously demonstrated that cyclosporin A (CsA) suppressed polyomavirus BK (BKV) replication. The role of cyclophilin A (CypA) and nuclear factor of activated T cells (NFAT) in CsA-imposed suppression of BKV replication was determined in this study. Results demonstrated that knockdown of CypA but not CypB significantly reduced BKV large T antigen (TAg) expression and BKV titer. Overexpression of CypA reversed CypA siRNA-induced inhibition in BKV TAg expression. In addition, CypA overexpression attenuated the suppressive effect of CsA on TAg expression, suggesting CypA implicated in CsA-mediated anti-BKV effect. Knockdown of NFATc3 abrogated TAg expression, while overexpression of NFATc3 promoted TAg expression and augmented BKV promoter activity. NFATc3 binding to the BKV promoter was verified by chromatin immunoprecipitation assay and electrophoretic mobility shift assay. Renal histology also displayed an increase in NFATc3 expression in tubulointerstitium of BKV-associated nephropathy. Furthermore, overexpression of NFATc3 rescued CsA-mediated inhibition of BKV load and TAg expression. A CsA analog, NIM811, which cannot block NFAT functionality, failed to suppress TAg expression. In conclusion, CypA and NFAT are indispensable in BKV replication. CsA inhibits BKV replication through CypA and NFAT, which may be potential targets of anti-BKV treatment.

Key words: Cyclophilin, cyclosporine, nuclear factor of activated T cell, polyomavirus BK

Abbreviations: BKV, polyomavirus BK; PVAN, polyomavirus-associated nephropathy; CMV, cytomegalovirus; CsA, cyclosporin A; CypA, cyclophilin A; HCV, hepatitis C virus; HIV, human immunodeficiency virus; TAg, large T antigen; NCCR, noncoding control region; NFAT, nuclear factor of activated T cells.

Received 25 May 2011, revised 29 March 2012 and accepted for publication 10 April 2012

Introduction

Polyomavirus BK (BKV) reactivation in renal transplant patients is a critical problem (1,2). It has been reported that 5–10% of the subjects develop polyomavirus-associated nephropathy (PVAN) (3) and up to 50% of the patients with PVAN eventually lose their allograft within 2–3 years (4). Tacrolimus and mycophenolate are associated with a higher incidence of PVAN compared with other immunosuppressants (5, 6). Cyclosporin A (CsA), a calcineurin inhibitor, has been commonly used for the prevention of allograft rejection after organ transplantation due to its selective function in inhibiting T cell immunity. In addition to immunosuppression, CsA can also inhibit replication of various viruses, including human immunodeficiency virus (HIV) type I, vaccinia virus, herpes simplex virus, hepatitis C virurs (HCV) and cytomegalovirus (CMV) (7–12). It has been reported that CsA can inhibit BKV replication in green monkey kidney cells (Vero E6 cells) (13). Similarly, we previously demonstrated that CsA suppressed BKV replication and its noncoding control region (NCCR) promoter activity in cultured human renal proximal tubular cells (14). Nevertheless, the mechanisms by which CsA inhibits BKV replication remain elusive.

Cyclophilin A (CypA) was isolated as a CsA-specific binding protein by Handschumacher et al. in 1984 (15). To date, seven major cyclophilin (Cyp) members including CypA, CypB, CypC, CypD, CypE, Cyp40 and CypNK have been identified in humans (16). CsA binds to cyclophilins to form a complex and subsequently inhibits phosphatase activity that is required for calcineurin activation. Through inhibition of calcineurin activation, CsA induces phosphorylation of nuclear factor of activated T cells (NFAT), which prevents translocation of NFAT from the cytoplasm into the nucleus, thereby blocking NFAT activity (17,18). Five NFAT